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IMMUNOCYTOCHEMICAL STUDY OF THE RED PIGMENT CONCENTRATING MATERIAL IN THE EYESTALK OF THE PRAWN *PALAEMON SERRATUS* PENNANT USING RABBIT ANTISERA AGAINST THE INSECT ADIPOKINETIC HORMONE

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Abstract

Using antisera produced against different parts of the insect adipokinetic hormone (AKH), it was possible to detect adipokinetic hormone-reactive peptides in the eyestalk of the prawn *Palaemon serratus*. Immunopositive staining was obtained in some neurosecretory cells of the medulla externa X organ (MEX), of the medulla terminalis X organ 2 (MTGX-2), in the lower part of the sinus gland, and in the nerve joining the medulla terminalis X organs to this neurohemal gland. The biological activity of synthetic AKH and RPCH (red pigment concentrating hormone) was tested on the movement of the red pigment in the chromatophores of the prawn and compared to the activity of extracts derived from immunoreactive tissue regions. The inhibiting effect of the AKH antibodies on the biological activity was ascertained by immuno-adsorption experiments. The results are discussed in relation to the molecular resemblance of AKH and RPCH. It is postulated that RPCH-material in the eyestalk of *Palaemon serratus* can be identified by using antisera against AKH-peptide. However, more than one type of immunoreactive RPCH/AKH-like peptide appears to be present in different groups of neurosecretory cells.

Introduction

Color changes in Crustacea are produced by the movement of pigments inside the different types of chromatophores. This pigment migration is regulated by a set of neurohormones, the chromatophorotropins. Among them the red pigment concentrating hormone (RPCH), extensively investigated in numerous species of prawns, supplies a model for the control mechanism of color adaptation in Crustacea.

Perkins (1928) and Koller (1928) provided the first experimental data pointing to the hormonal influence of the eyestalk on the crustacean color changes. Since then innumerable studies have given evidence for the physiological role of the eyestalk chromatophorotropins, particularly of RPCH (see reviews in Carlisle and Knowles,

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According to personal communication comparable results have been obtained by S. Mangerich, H. Dircksen, and R. Keller in their manuscript "Immunocytochemical identification of putative red pigment concentrating hormone-containing structures in the central nervous system of decapod crustaceans" (in press).

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1959; Scholz, 1961; Fingerman, 1963; Bellon-Humbert, 1970; Noël, 1981). Presently, isolation and characterization of RPCH have allowed the production of synthetic analogs. These preparations have fostered the study of structure-activity relationships (Otsu, 1965; Fernlund, 1968, 1974; Fernlund and Josefsson, 1968, 1972; Fingerman, 1973; Skorkowski, 1973; Josefsson, 1975; Carlsen *et al.,* 1976; Jaffe *et al.,* 1982). Furthermore, the purification of pigment concentrating factors from the corpora cardiaca, neurohemal centers in insects, led to the isolation and characterization of the insect adipokinetic hormones, AKH (I) and AKH (II) (Goldsworthy *et al.,* 1972; Stone *et al.,* 1976). These two neuropeptides are close structural analogs to the crustacean RPCH (Mordue and Stone, 1978; Josefsson, 1983). Recently, one of the authors (H.S.) and his colleagues identified AKH-like material in the locust and other insects using C-terminal and N-terminal specific antisera to AKH (I) (Schooneveld *et al.,* 1983, 1985a, b, 1986).

In spite of the detailed knowledge of the RPCH molecule and its physiological function, information about the cells synthetizing the RPCH material in the eyestalk of Crustacea was not available until now. Consequently the pattern of cellular activities such as synthesis, processing, storage and release is unknown. The suggestion that a neurosecretory cell group in the MEX organ of the eyestalk may be a production center for RPCH active material was provided some years ago by two of us (C.B. and F.v.H) using extirpation- and injection-procedures (Bellon-Humbert *et al.,* 1981).

The objective of the present study was to collect more information about RPCH and its presence in the neurosecretory system of the eyestalk from the prawn *Palaemon serratus* using C-terminal and N-terminal specific antisera to AKH (I).

Materials and Methods

Collection and maintenance of prawns

Adult prawns, *Palaemon serratus* Pennant, were collected in summer and in winter in the Bay of Concarneau (France). They were maintained in running seawater at the normal season temperatures (17°C in summer—11°C in winter), with the natural photoperiod. The animals were fed daily with synthetic food pellets and fresh mussels. For each experiment, prawns were selected according to their size (55–60 mm) and molting cycle (intermolt C-stage).

Immunocytochemical experiments

Between 9:00 and 10:00 a.m., eyestalks were removed from prawns in C stage of their molting cycle. They were fixed in Bouin-Hollande fluid containing 10% of a saturated aqueous solution of sublimate, then dehydrated, cleared according to the conventional histological procedure, and embedded in Histomed (melting point 58°C). Serial sections 7 μm thick (longitudinal and transversal) were deparaffinized, washed in Lugol and a hyposulphite solution, and rinsed in distilled water before equilibration in 0.05 *M* Tris-HCl buffered saline, pH 7.6. Then the sections were immunocytochemically stained by the peroxydase-antiperoxydase (PAP) method (Sternberger, 1979), using 4-Cl-1-naphthol as a marker for the peroxydase activity. The best results were obtained with the following incubation procedure: (1) Normal goat serum (dilution 1:5): 10 min incubation; (2) Anti-AKH-serum (code 241 or 433) (dilution 1:750–1:1000): incubation for 24 to 48 h at 4°C; wash; (3) Goat anti-rabbit IgG (H + L) serum (dilution 1:20): 20 min; wash; (4) PAP complex (dilution 1:50): 30 min; wash; (5) Substrate incubation (100 mg 4-Cl-1-naphthol in 0.05 *M* Tris-HCL,

pH 7.6, containing 0.005% H_2O_2): 7 min; wash in distilled water; (6) Mounting in water medium.

The primary antisera were produced in rabbits against a complex of synthetic (Tyr^1) AKH and thyroglobulin (code 241/3-6-82: Schooneveld *et al.*, 1983) and against AKH (1–4) also conjugated to thyroglobulin (code 433: Schooneveld *et al.*, 1985b). The former antiserum is referred to as a C-terminal specific, the latter as an N-terminal specific antiserum (Schooneveld *et al.*, 1986). The second antiserum [GAR IgG (H+L)] and the PAP complex were obtained from Nordic (Tilburg, The Netherlands). The specificity of the immunocytochemical staining method was tested by successively substituting each of the incubation reagents in the normal procedure for buffer and by using the anti-AKH serum preadsorbed with 10 nmol synthetic AKH or synthetic RPCH/ml. The controls were carried out on sections adjacent to those stained with the complete immunoenzyme cytochemical procedure. Sometimes azan-staining was used to identify the immunoreactive cells and structures on the sections.

Physiological experiments

To obtain more information about the biological activity of the red pigment concentrating (RPC) material in the different eyestalk structures of the prawns, injection experiments were carried out. The erythrophorotropic activity of the following tissue extracts was tested: sinus gland; medulla terminalis X-organs (as the $MTGX_1$ and $MTGX_2$ are not easily removed from the circumscribed cells, the medulla terminalis was extracted as a whole); medulla externa X-organ (MEX); organ of Bellonci; medulla interna, externa and lamina ganglionaris material; whole eyestalk. Synthetic AKH and synthetic RPCH (Peninsula Labs) were also tested. In addition the biological activity was compared in sample series non treated and pre-treated with the rabbit anti-AKH-serum code 241.

Preparation of the tissue extracts, dilution series of samples, and injection procedure. The aforementioned eyestalk structures were dissected from the eyestalks of large prawns in molting stage C and collected in icy-cold saline (distilled water + filtered seawater 1:1). After homogenization in a microglass grinder, the extracts were centrifuged (3000 × *g*; 20 min; 4°C) and the supernatants were collected and lyophilized. For each extract a dilution series of 1, 0.1, 0.01, and 0.001 equivalent/10 μl saline was prepared. The two synthetic peptides were tested in a dilution series of 1 pmol, 100 fmol, 10 fmol, and 1 fmol/10 μl saline. All samples were injected into the abdominal region between the muscles and the cuticle from eyestalkless intermolt prawns, using a thin needle microsyringe. Eyestalk ablation was carried out 24 h before injection, so that pigments were fully expanded (index 5).

Preincubation of the samples with antisera. To study the effect of the immunochemical reaction of the anti-AKH sera on the biological activity of selected samples, 1 μl undiluted antiserum (code 241) was added to 100 μl of the dilution series of synthetic AKH, RPCH and two tissue samples (MEX organ extract and sinus gland extract). These solutions were gently mixed and incubated for 1 h at 37°C then 24 h at 4°C. Afterwards, they were centrifuged (20,000 × *g*; 5 min; 4°C), the supernatants collected and used for injection experiments in comparison to the non-treated samples.

Chromatophore index estimation. The two types of erythrophores, the large ones drawing a specific pattern of bands and the small ones, scattered in their intervals, were examined. The small erythrophores were the most reactive. In the data pre-

sent in here only their activities were retained. Measurements were recorded in Panouse's scale (1946) using 6 stages characterizing the pigment dispersion grade (0: full concentration, punctiform chromatophore; 1: irregular shaped chromatophore; 2: stellate chromatophore with a few large chromorhizae; 3: sea urchin-like aspect with bifurcated chromorhizae; 4: highly bifurcated and fine chromorhizae, chromatophores still distinct; 5: full dispersion, adjacent chromatophores with confused chromorhizae). The measurements were always recorded in the dorsal area of the first abdominal segment on pools of 10 animals for each sample and at intervals of 10 min up to 60 min. All the physiological experiments were performed between 10 and 12 a.m., in natural daylight conditions and on a white background.

Results

Immunocytochemical observations

Immunopositive reactions with both anti-AKH sera were detected in some neurosecretory cells of the medulla externa and medulla terminalis, in the sinus gland, as well as in the nervous tract, starting in the medulla terminalis X organ 2 and running up to the neurohemal region (Fig. 1). While some immunoreactive cells were localized at the base of the medulla terminalis, most of them were found in two neurosecretory cell groups: the medulla externa X organ or MEX and the medulla terminalis X organ 2 or MTGX-2.

In the MEX, on the dorsal side of the eyestalk, 1 to 4 cells were strongly stained with the anti-AKH serum code 241 and faintly with the anti-AKH serum code 433. These cells are characterized by a large size (diameter about 30–32 μm), a round and large nucleus (diameter about 13–15 μm), and a rough granular cytoplasm. They are localized inside the organ and are covered by small neurons (Fig. 2).

In the MTGX-2, on the ventral side of the eyestalk, a variable immunostaining with sera 241 and 433 was observed in 1 to 5 cells. These cells are located between the proximal part of the organ of Bellonci and the cells synthetizing the hyperglycemic hormone or CHH (Van Herp *et al.*, 1984). The diameter of the cells reacting with the two antisera ranges from 20 to 30 μm. But sometimes, 1 to 2 larger cells (diameter $\geqslant$30 μm) in the center of the MTGX-2 were also immunoreactive (Fig. 3).

The immunostaining of the sinus gland was obvious and striking: only the inner and lower part of the gland (proximal part) contains axon terminals stained with both anti-AKH sera (Figs. 4, 5). These terminals belong to neurons in the MTGX-2 area as it was possible to visualize partial portions of the nerve joining the MTGX-2 and the sinus gland (Figs. 4, 6). In the medulla terminalis, in the medulla externa, and in the lamina ganglionaris, a positive reaction to AKH antibodies of the C terminal serum was observed in grouped nerve fibers (Fig. 1). The perikarya of these nerves could not be identified with certainty.

After successively substituting each of the incubation reagents with buffer or after incubation with anti-AKH serum adsorbed with AKH or RPCH, no elements were stained. These control data are summarized in Table I. Because the cells at the basis of the medulla terminalis (Fig. 1) remain positive after incubation with normal rabbit serum, their immunoreaction was interpreted as non specific.

Physiological effects of tissue extracts and peptides

In an attempt to relate the presence of immunoreactive material to well characterized physiological parameters, the RPCH activity of the different immunoreactive

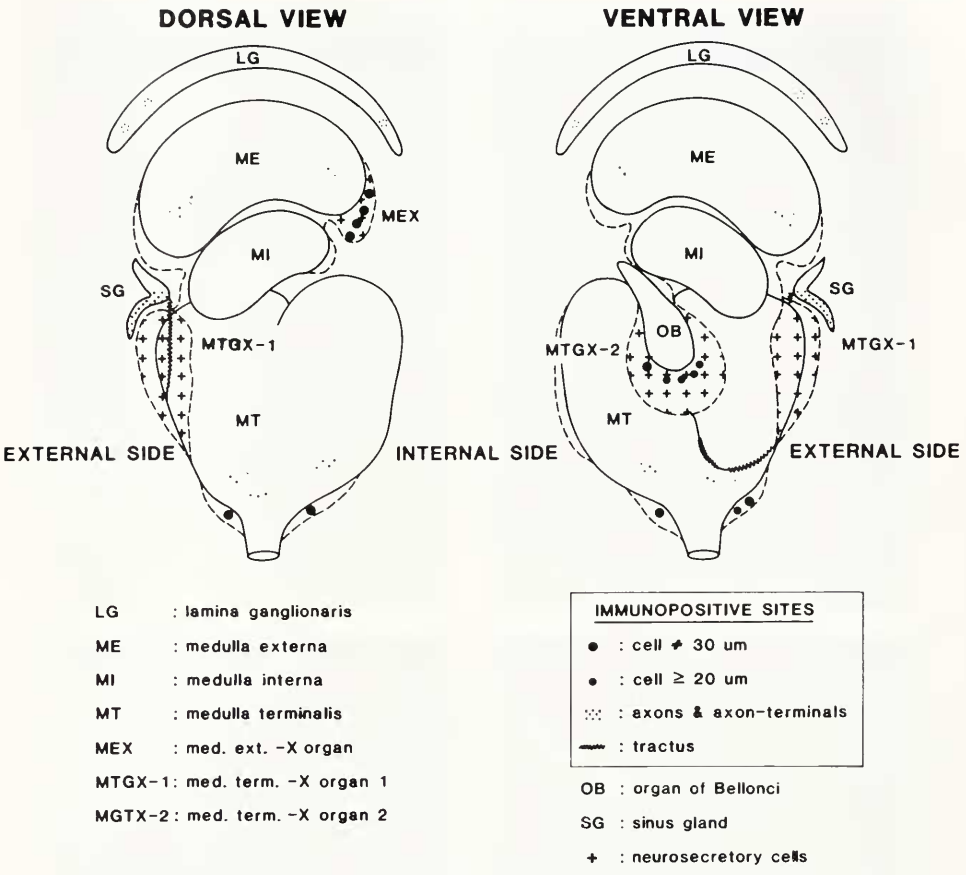
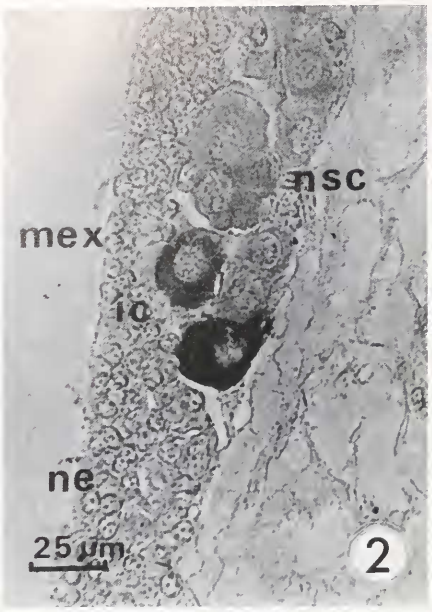


FIGURE 1. General organization of the eyestalk of *Palaemon serratus* with the indication of the immunopositive sites for anti-AKH sera.

structures of the eyestalk was investigated. Extracts of total eyestalks or of selected organs were prepared (see Materials and Methods) and one organ equivalent or less was injected into individual eyestalkless prawns. In these prawns the red pigment was maximally dispersed. All bioassay results were compared to the chromatophorotropic activity of the total eyestalk (Figs. 7A, B). MEX extracts were more active than MT extracts (MTGX-1 and 2) and comparable to the activity of the whole medullae (LG, ME, MI). Sinus gland extracts were very potent and approached the activity of total eyestalk extracts. A strong concentration of the red pigment was observed after the injection of extracts of the organ of Bellonci, in spite of the lack of reaction with the anti-AKH sera.

The MEX and the sinus gland were selected for answering the question if the physiological response of extracts showed a dose dependency. As Figures 8A and B show, this is clearly the case. The intensity of the response is compared to that of pure RPCH and AKH, tested simultaneously (Figs. 8C, D). It appeared that the biological activity of 1 sinus gland was comparable to that of 1 pmol RPCH and superior to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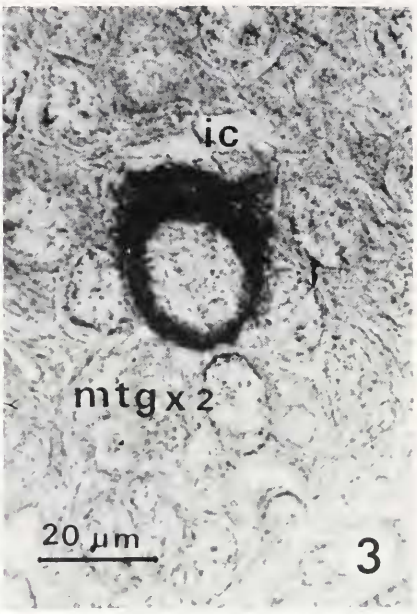
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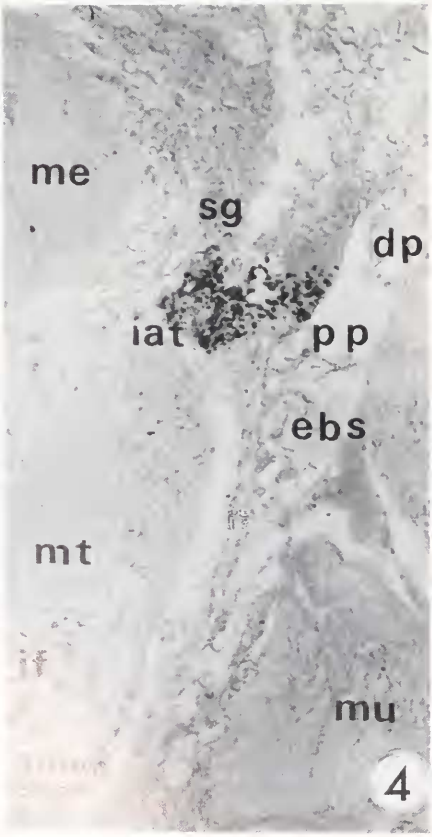
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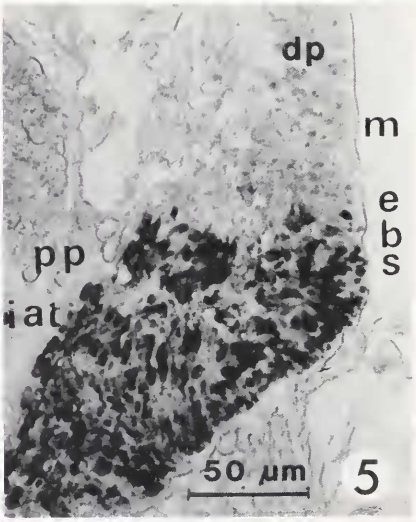
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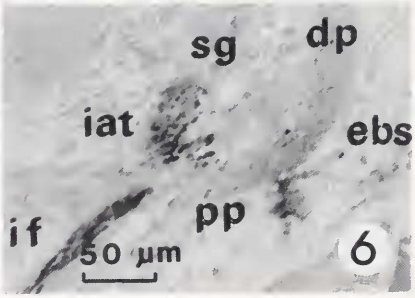
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TABLE I

Immunostaining in the eyestalk of the prawn Palaemon serratus *after incubation with anti-AKH sera (code 241 and code 433) and control tests*

Antiserum	Dilution	Incubation	Immunoreactive sites			
			SG	MEX	MTGX-2	Tractus
Code 241	1/750	24 h	+++	(+)	+	+
Code 241	1/750	48 h	+++	+++	++	++
Code 433	1/1000	24 h	++	−	+	+
Code 433	1/1000	48 h	++	(+)	+	+
NRS		24 h	−	−	−	−
AKH/anti-AKH (code 241)	1/750	24 h	(+)	−	(+)	−
RPCH/anti-AKH (code 241)	1/750	24 h	−	−	−	−
RPCH/anti-AKH (code 433)	1/1000	24 h	(+)	−	−	−

+++: very strong staining; ++: intermediate staining; +: weak staining; (+): doubtful staining; −: no staining; NRS: normal rabbit serum.

that of 1 pmol AKH while the activity of 1 MEX organ corresponded approximately to 10 fmol RPCH or 100 fmol AKH.

The chromatophorotropic activity of organs and synthetic AKH and RPCH after preincubation with the anti-AKH serum (code 241) was tested to obtain additional evidence that the immunoreactive structures are in fact responsible for the RPCH effects of tissue homogenates. Figures 9A and B show that immunoprecipitation of AKH-like material did not decrease the activity of the sinus gland extracts, when the concentration of 1 and 0.1 structure equivalent/10 μl saline was used. MEX extracts lost their biological activity almost completely if concentration comprised between 1 or 0.1 MEX equivalent/10 μl saline were pretreated with antiserum (Figs. 10A, B). The synthetic hormones RPCH and AKH did not lose their activity by this procedure. No significant effect on the RPCH activity was detected with preincubated RPCH, whatever the concentration was; a slightly perceptible effect was only noticed for the preincubated AKH, when the concentration was 100 fmol (results not shown).

FIGURE 2. Detail of the neurosecretory cells (nsc) of the MEX-organ (mex), showing the situation of the immunopositive cells (ic) bordered by neurons (ne).

FIGURE 3. Large immunopositive cell inside the MTGX-2 organ (mtgx2). Note the very strong reaction with the anti-AKH serum.

FIGURE 4. Longitudinal section of a right eyestalk, showing the sinus gland (sg) with its two regions: proximal (pp) and distal (dp) parts. Immunopositive axon terminals (iat) are located in the proximal part. Note also the immunopositive fibres (if) of the nerve joining the sinus gland to the MTGX-2 through the medulla terminalis (ebs: external blood sinus; mu: muscle).

FIGURE 5. Detail of a longitudinal section of the sinus gland. Note the immunopositive axon terminals in the proximal part of the gland in contrast to the immunonegative axon terminals of the distal part (m: outer membrane of the gland).

FIGURE 6. View of the sinus gland with immunopositive fibers and axon terminals of the nerve originated from MTGX-2.

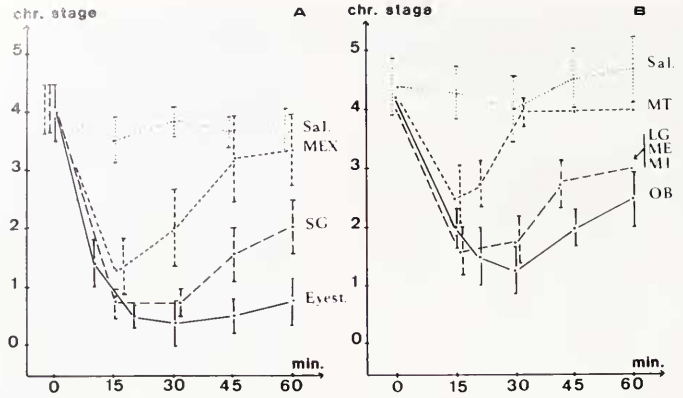

FIGURE 7A, B. Effect of extracts of different eyestalk structures on the red pigment concentration in the small erythrophores of the prawn (Sal: saline; Eyest.: eyestalk; LG, ME, MI: lamina ganglionaris + medullae externa and interna; MEX: X-organ of the medulla externa; MT: medulla terminalis without OB; OB: organ of Bellonci; SG: sinus gland).

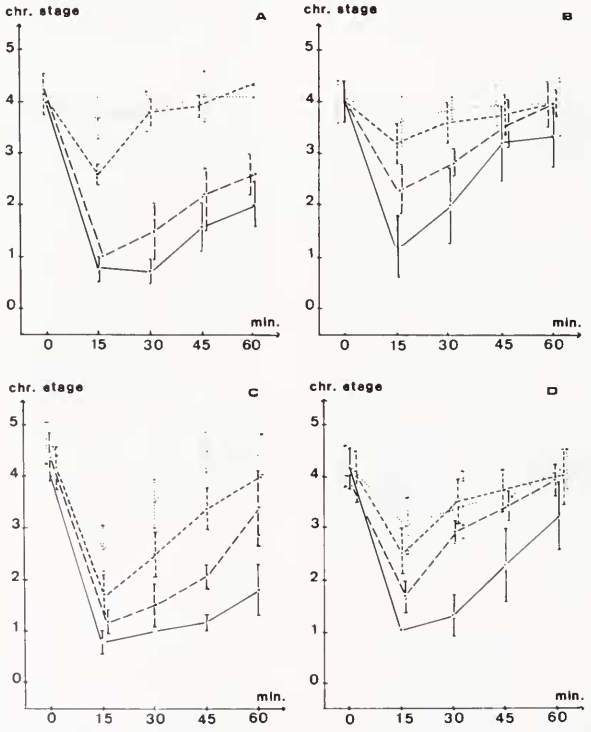

FIGURE 8. Red pigment concentrating activity of the sinus gland (A) and MEX (B) extracts, compared to the activity of the synthetic peptides RPCH (C) and AKH (D). A and B (——: 1 structure equivalent/10 μl; – – –: 0.1 structure equivalent/10 μl; -----: 0.01 structure equivalent/10 μl; · · · ·: 0.001 structure equivalent/10 μl) compared respectively to C and D (——: 1 pmol hormone/10 μl; – – –: 100 fmol hormone/10 μl; -----: 10 fmol/10 μl; · · · ·: 1 fmol/10 μl).

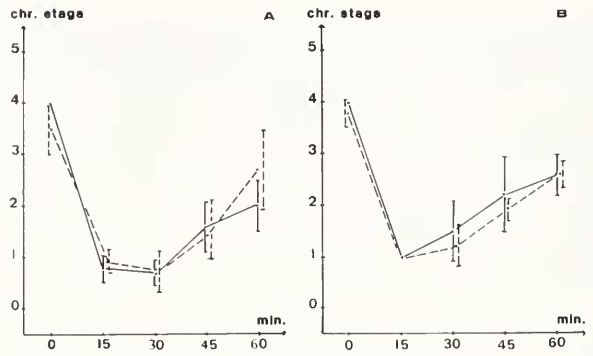

FIGURE 9. Red pigment concentrating activity of the sinus gland extracts, pre-incubated with the anti-AKH serum code 241 (——: untreated extracts; – – –: pre-incubated extracts; A: 1 SG equivalent/10 μl; B: 0.1 SG equivalent/10 μl).

DISCUSSION

The detection of RPCH/AKH-like material in the eyestalk of the prawn *Palaemon serratus* was based on the chemical relationship which exists between the crustacean RPCH and the insect hormone AKH. The molecular structure of the AKH (Stone *et al.*, 1976) closely resembles that of the RPCH (Fernlund, 1974) and these two substances "reproduce each other's biological effects when cross-tested on members of the two arthropod groups" (Mordue and Stone, 1976). These properties suggested the use of two different anti-AKH sera to reveal the sites producing and storing RPCH/AKH-like material in the eyestalk of the prawn and to compare the biological activities of tissue extracts with those of synthetic AKH and RPCH.

The antisera used were raised in rabbits against (Tyr^1) - AKH (Tyr-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2) coupled through its N-terminal to thyroglobulin (code 241) and against the AKH (1–4) peptide (pGlu-Leu-Asn-Phe) also coupled to thyroglobulin through its C-terminal (code 433) (Schooneveld *et al.*, 1983, 1985b,

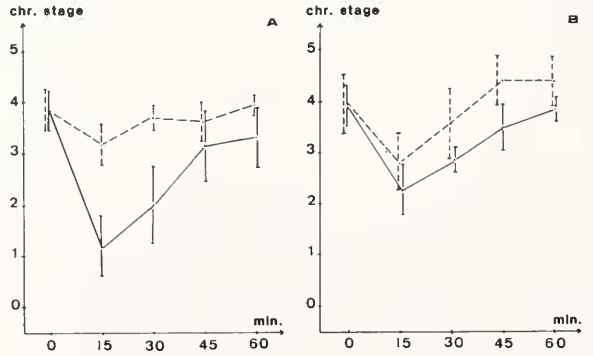

FIGURE 10. Red pigment concentrating activity of the MEX extracts, pre-incubated with the anti-AKH serum code 241 (——: untreated extracts; – – –: pre-incubated extracts; A: 1 MEX equivalent/10 μl; B: 0.1 MEX equivalent/10 μl).

1986). With both antisera we could visualize the same morphological structures: some neurosecretory cells in the MEX and MTGX-2 organs, one part of the sinus gland, and a tract. The homologous staining in the mentioned structures implies that neuropeptidergic material containing the common amino acid sequence (-Leu-Asn-Phe-) reacts with the antisera. As the RPCH molecule in prawns has the same amino acid sequence in the same position (pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2), it is possible that the material identified by the antisera belongs to the PCH family. The fact that the immunocytochemical reaction of the anti-AKH sera is mostly inhibited with synthetic AKH as well as with synthetic RPCH, gives additional support to this hypothesis. However, at this time it cannot be excluded that other molecules with similar epitopes react positively with the antisera.

The synthesis of AKH-like material in the cells of the MEX was expected: we already mentioned the progressive dispersion of the red pigment when this organ was removed and the concentrating effect induced by the MEX-extracts (Bellon *et al.*, 1981). We were able to distinguish in this organ four cell types according to their morphological characteristics and staining properties (Van Herp *et al.*, 1977). The immunoreactive cells can be classified as α or β cells because of their size, nucleus shape, and location.

AKH-like material was also detected in some neurosecretory cells of the medulla terminalis, chiefly in the MTGX-2 organ. This organ is considered to be the main source of neurohormones in the eyestalk, since the axons originating from the neurosecretory cells form the only important nerve running to the sinus gland. These cells occur in small numbers and have variable size and secretion content. Apparently, these cells can be classified as ϵ and π cells, according to Van Herp *et al.* (1977). The differences in stainability may be related to the cellular rhythms in synthetic activity (diurnal and molting cycles), but also to the background adaptation and illumination conditions. We noticed that the strongest immunocytochemical reaction was observed in some of the largest cells of the MTGX-2 organ.

The immunoreaction in the sinus gland and in the tract with the AKH antisera implies that the immunoreactive material is carried by the nerve tract from the neurosecretory cells of the medulla terminalis and mainly stored in the lower part of the gland. The restricted immunoreaction in the sinus gland points to the unequal distribution of axon terminals, containing the neurosecretory granules. In fact, on the ultrastructural level, there was no difference in the distribution of the five neurosecretory granule types previously described (Strolenberg *et al.*, 1977). The electron density and the granule size therefore cannot be used to differentiate neurosecretory products from each other. Immunocytochemical methods may help further differentiate granule populations in the electron microscope.

The lack of immunological tracing of a nerve tract originating from the MEX organ to the sinus gland and the presence of immunoreactive fibers in the lamina ganglionaris set the problem of the release of AKH-like material produced in the MEX neurosecretory cells and of the origin of the fibers observed in the upper ganglia. It is possible that this statement reflects a functional difference between the peptidergic cells in the MEX and MTGX-2 organs and that a different mode of release is correlated respectively to neuromodulator and neurohormonal functions. Moreover, during embryogenesis, the medulla externa and medulla terminalis develop differently (Elofsson, 1969; Bellon-Humbert, 1985) and a double chiasma, crossing the axons, affects the medulla externa.

Special attention has been paid to the question of whether the material demonstrated immunocytochemically was responsible for the physiological effects on chro-

matophore activation of tissue extracts containing such immunoreactive material. If the sinus gland and MEX extracts give clear and expected results, the medulla terminalis was found less active, when the organ of Bellonci was removed. The red pigment concentration induced by extracts of this last organ was carefully controlled. However, this effect remains difficult to explain. Immunocytochemically no AKH-like material is detectable. Analogs to vasopressin and neurophysin (Van Herp and Bellon-Humbert, 1982) and to 5-HT (unpub. results), have no effect on the red pigment concentration. Although we cannot exclude that a few small cells, containing AKH-like material and which are closely in contact with the proximal part of the organ of Bellonci, are injected together with this organ. This can also explain the relatively small effect of the medulla terminalis extracts.

We attempted also to eliminate the physiological factor from the tissue extracts by immunoprecipitation with the anti-AKH serum (code 241) in watery incubation medium. The experiments were performed with the tissues containing appreciable amounts of biologically active material, *i.e.*, the sinus gland and the MEX of the eyestalk. Using fixed amounts of antiserum, the doses of sinus gland extract assayed did not lose their RPC effects. The dose of antibody used (1 μl) presumably was too low to inactivate larger quantities of active factor. This factor could well be authentic RPCH.

The results with the MEX were promising. We showed that the largest dose of MEX extract was fully inactivated by the antiserum. Why the low dosage retains some of its activity remains to be investigated. A possible explanation is that low concentrations of immune complexes are less easily removed from the incubation medium by our centrifugation procedure. From the data obtained in our experiments using synthetic peptides, we deduce that the biological active sites, consisting in the tryptophan residue of the RPCH peptide (Carlsen *et al.*, 1979; Josefsson, 1983) are possibly not always blocked by the anti-AKH serum. Our results with synthetic RPCH and AKH show that no loss of activity can be observed if solutions of peptides are pretreated with anti-AKH serum (code 241). This would be in agreement with earlier observations with immunodiffusion experiments indicating that no precipitation of AKH-IgG complexes can be visualized in gels (Schooneveld, unpub. result). In recent immunoprecipitation tests using goat anti-rabbit IgG serum to precipitate the entire complex, we observed that the primary complex (synthetic RPCH anti-AKH) is only precipitable by addition of a second antibody as GAR (Van Herp, unpub. result). If the first complex indeed remains in solution, the failure to inactivate the biological activity of the peptide perhaps can be attributed to the reversibility of the formation of this antigen-antibody complex. The peptide may then have the option to associate either with the antibody or with the peptide receptors in tissues. The latter bond may be the stronger of the two, leaving the antibodies (partially) unoccupied.

The fact that MEX extracts could be inactivated by preincubation with antiserum implies that the immunoreactive substance in this organ might be related to a physiologically active factor. But whether this factor is RPCH or some other related molecule remains to be investigated by more elaborate immunoprecipitation experiments and chemical analysis. It should be born in mind that insects appear to contain a wide variety of AKH-related peptides in endocrine centers as well as in neurons of the central nervous system. Using the antisera also used here, immunoreactive substances proved to differ from each other in certain staining properties (Schooneveld *et al.*, 1986). Moreover, different peptides related to AKH, including RPCH, were able to mimic AKH in the locust bioassay (Siegert *et al.*, 1985). It cannot be excluded

the case that a similar family of related peptides also occurs in our prawn. One indication of a peptide diversity is the observation that the cells in the MEX hardly stain with antiserum 433, whereas a strong staining is obtained with antiserum 241 (Table I).

The partial adsorption of MEX extracts with the antiserum (due to the precipitation of the immunocomplex sometimes visualized after centrifugation) is another indication that the AKH-like material in the MEX-organ is different from that in the sinus gland. In this last structure, the erythrophoretic activity can be related to a small neuropeptide, as shown by the comparable physiological effects of the synthetic hormone, while the MEX-material can be a large polypeptide, precipitable by the antiserum.

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LITERATURE CITED

BELLON-HUMBERT, C. 1970. Les changements de couleur des crustacés. Pp. 259–317 in *Hormones et Comportement, Sér. 14, Problémes Actuels d'Endocrinologie et de Nutrition,* H. P. Klotz, ed. Expansion Scientifique Française, Paris.

BELLON-HUMBERT, C. 1985. Développement embryonnaire de *Palaemon serratus:* résultats préliminaires. Pp. 181–194 in *Actes de Colloques no. 1, Bases Biologiques de l'Aquaculture,* IFREMER, ed. Montpellier 12–16 décembre 1983. SDP, Brest.

BELLON-HUMBERT, C., F. VAN HERP, G. E. C. M. STROLENBERG, AND J. M. DENUCÉ. 1981. Histological and physiological aspects of the medulla externa X organ, a neurosecretory cell group in the eyestalk of *Palaemon serratus* Pennant (Crustacea Decapoda Natantia). *Biol. Bull.* **160:** 11–30.

CARLISLE, D. B., AND F. G. W. KNOWLES. 1959. *Endocrine Control in Crustaceans.* Cambridge University Press, Cambridge. 120 pp.

CARLSEN, J., M. CHRISTENSEN, AND L. JOSEFSSON. 1976. Purification and chemical structure of the red pigment-concentrating hormone of the prawn *Leander adspersus. Gen. Comp. Endocrinol.* **30:** 327–331.

CARLSEN, J., M. CHRISTENSEN, AND L. JOSEFSSON. 1979. Preparation of an ^{125}I-labelled red pigment concentrating hormone analog. *Anal. Biochem.* **92:** 46–54.

ELOFSSON, R. 1969. The development of the compound eyes of *Penaeus duorarum* (Crustacea Decapoda) with remarks on the nervous system. *Z. Zellforsch.* **97:** 323–350.

FERNLUND, P. 1968. Chromactivating hormones of *Pandalus borealis.* Bioassay of the red-pigment concentrating hormone. *Mar. Biol.* **2:** 13–18.

FERNLUND, P. 1974. Synthesis of the red pigment concentrating hormone of the shrimp *Pandalus borealis. Biochim. Biophys. Acta* **371:** 304–311.

FERNLUND, P., AND L. JOSEFSSON. 1968. Chromactivating hormones of *Pandalus borealis.* Isolation and purification of the red pigment-concentrating hormone. *Biochim. Biophys. Acta* **158:** 262–273.

FERNLUND, P., AND L. JOSEFSSON. 1972. Crustacean color change hormone: amino acid sequence and chemical synthesis. *Science* **177:** 173–175.

FINGERMAN, M. 1963. *The Control of Chromatophores.* Pergamon Press, Oxford-Londres-New York-Paris. 184 pp.

FINGERMAN, M. 1973. Behavior of chromatophores of the fiddler crab *Uca pugilator* and the dwarf crayfish *Cambarellus shufeldti* in response to synthetic *Pandalus* red pigment-concentrating hormone. *Gen. Comp. Endocrinol.* **20:** 589–592.

GROSPHY, G. J., W. MORDUE, AND J. GUTHKELCH. 1972. Studies on insect adipokinetic hormones. ... *Comp. Endocrinol.* **18:** 545–551.

... LOEB, D. K. HAYES, AND N. HOLSTON. 1982. Rapid isolation of nanogram amounts of ... erythrophore concentrating hormone from invertebrate nerve tissue by RP-HPLC. *J. ... hromatog.* **5:** 1375–1390.

JOSEFSSON, L. 1975. Structure and function of crustacean chromatophorotropins. *Gen. Comp. Endocrinol.* **25:** 199–202.

JOSEFSSON, L. 1983. Invertebrate neuropeptide hormones. *Int. J. Peptide Protein Res.* **21:** 459–470.

KLEINHOLZ, L. H. 1961. Pigmentary effectors. Pp. 133–169 in *The Physiology of Crustacea,* II, T. H. Waterman, ed. Academic Press, New York-London.

KOLLER, G. 1928. Versuche über die inkretorischen Vorgänge beim Garnelenfarbwechsel. *Z. Vergl. Physiol.* **8:** 601–612.

MORDUE, W., AND J. V. STONE. 1976. Comparison of the biological activities of an insect and a crustacean neurohormone that are structurally similar. *Nature* **264:** 287–289.

MORDUE, W., AND J. V. STONE. 1978. Structure and metabolism of adipokinetic hormone. Pp. 487–490 in *Comparative Endocrinology,* P. H. Gaillard and H. H. Boer, eds. Elsevier North-Holland, Amsterdam.

NOËL, P. Y. 1981. Hormonal control of chromatophores in Crustacea. *J. Sci. Ind. Res.* **40:** 267–276.

OTSU, T. 1965. Component amino-acids of chromatophore concentrating hormones from Decapoda Crustacea. *Naturwissenschaften* **52:** 187–188.

PANOUSE, J. B. 1946. Recherches sur les phénomènes humoraux chez les Crustacés. L'adaptation chromatique et la croissance ovarienne chez la crevette *Leander serratus. Ann. Inst. Oceanogr.* **32:** 65–147.

PERKINS, E. B. 1928. Colour changes in crustaceans, specially in *Palaemonetes. J. Exp. Zool.* **50:** 71–95.

SCHOONEVELD, H., G. I. TESSER, J. A. VEENSTRA, AND H. M. ROMBERG-PRIVEE. 1983. Adipokinetic hormone and AKH-like peptide demonstrated in the corpora cardiaca and nervous system of *Locusta migratoria* by immunocytochemistry. *Cell Tissue Res.* **230:** 67–76.

SCHOONEVELD, H., H. M. ROMBERG-PRIVEE, AND J. A. VEENSTRA. 1985a. Adipokinetic hormone-immunoreactive peptide in the endocrine and central nervous system of several insect species. A comparative immunocytochemical approach. *Gen. Comp. Endocrinol.* **57:** 184–194.

SCHOONEVELD, H., AND J. A. VEENSTRA. 1985b. Cells and neurons containing various adipokinetic hormone immunoreactive substances. Pp. 425–434 in *Neurosecretion and the Biology of Neuropeptides,* H. Kobayashi, H. A. Bern, and A. Urano, eds. Springer-Verlag, Berlin.

SCHOONEVELD, H., H. M. ROMBERG-PRIVEE, AND J. A. VEENSTRA. 1986. Immunocytochemical differentiation between adipokinetic hormone (AKH)-like peptides in neurons and glandular cells in the corpus cardiacum of *Locusta migratoria* and *Periplaneta americana* with C-terminal and N-terminal specific antisera to AKH. *Cell Tissue Res.* **243:** 9–14.

SIEGERT, K., P. MORGAN, AND W. MORDUE. 1985. Primary structures of locust adipokinetic hormones II. *Biol. Chem. Hoppe-Seyler* **366:** 723–727.

SKORKOWSKI, E. F. 1973. Chromatophorotropic neurohormones of the shrimp *Crangon crangon.* Partial purification of the red pigment concentrating hormone. *Mar. Biol.* **19:** 149–152.

STERNBERGER, L. A. 1979. *Immunocytochemistry,* 2nd ed. John Wiley and Sons, New York. 354 pp.

STONE, J. V., W. MORDUE, K. E. BATLEY, AND H. R. MORRIS. 1976. Structure of locust adipokinetic hormone, a neurohormone that regulates lipid utilisation during flight. *Nature* **263:** 207–211.

STROLENBERG, G. E. C. M., C. BELLON-HUMBERT, F. VAN HERP, AND A. VAN WORMHOUDT. 1977. Etude ultrastructurale des granules de neurosecrétion dans la glande du sinus de *Palaemon serratus* (Pennant). *C. R. Acad. Sci. D* **284:** 123–126.

VAN HERP, F., C. BELLON-HUMBERT, J. T. M. LUUB, AND A. VAN WORMHOUDT. 1977. A histophysiological study of the eyestalk of *Palaemon serratus* (Pennant) with special reference to the impact of light and darkness. *Arch. Biol.* **88:** 257–278.

VAN HERP, F., AND C. BELLON-HUMBERT. 1982. Localisation immunocytochimique de substances apparentées à la neurophysine et à la vasopressine dans le pédoncule oculaire de *Palaemon serratus* Pennant (Crustacé Décapode Natantia). *C. R. Acad. Sci. D* **295:** 97–102.

VAN HERP, F., A. VAN WORMHOUDT, W. A. J. VAN VENROOY, AND C. BELLON-HUMBERT. 1984. Immunocytochemical study of Crustacean Hyperglycemic Hormone (CHH) in the eyestalks of the prawn *Palaemon serratus* (Pennant) and some other Palaemonidae, in relation to variations in the blood glucose level. *J. Morphol.* **182:** 85–94.